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Smooth Muscle-Specific Expression of Neurotrophin-3 in the Embryonic and Neonatal Gastrointestinal Tract of the Mouse

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ABSTRACT (256 words)

Neurotrophin-3 (NT-3), which is essential for the survival of a large proportion of vagal sensory neurons, is expressed in the developing gastrointestinal (GI) tract where it could contribute to this survival, to other aspects of vagal afferent development, and to the development of spinal afferents, postganglionic sympathetic neurons and intrinsic neurons. Identifying the functions of this peripheral NT-3 requires a detailed understanding of the localization and timing of its expression in the developing GI tract. Therefore, X-gal staining of embryos and neonates expressing the *lacZ* reporter gene from the NT-3 locus was used to characterize the spatiotemporal pattern of NT-3 expression during development. NT-3 expression in the stomach, and intestines was largely restricted to smooth muscle of the outer GI wall and associated blood vessels. However, expression also occurred in the stomach lamina propria and esophageal epithelium. NT-3 expression occurred in GI wall mesenchyme by embryonic day (E)12.5 and became restricted to smooth muscle and lamina propria by E15.5 as these tissues differentiated, whereas expression in blood vessels and esophageal epithelium was first observed at E15.5. Immunohistochemical detection of β-galactosidase and cell type markers suggested myenteric elements, including neurons, glial cells, neural and glial precursors, and interstitial cells of Cajal did not express NT-3. Thus, NT-3 expression in the GI tract was largely restricted to smooth muscle at ages when vagal axons grow into the GI tract and vagal mechanoreceptors form in smooth muscle, consistent with roles in these processes, in vagal sensory neuron survival, and in development of intrinsic and other extrinsic GI wall innervation.

INTRODUCTION

[Need to incorporate discussion of extrinsic and intrinsic myenteric elements that express trk and p75 receptors during development as targets for secreted NT-3 from smooth muscle.]

Neurotrophin-3 (NT-3) is one of four members of the mammalian neurotrophin family of secreted proteins. The high affinity receptor for NT-3 is the receptor tyrosine kinase, trkC [Kaplan, 1991 #1183; Klein, 1991 #1184; Cordon-Cardo, 1991 #1182; Berkemeier, 1991 #1157; Glass, 1991 #1181; Soppet, 1991 #1173; Squinto, 1991 #1172; Lamballe, 1991 #1180], although it can also activate trkA or trkB [Berkemeier, 1991 #1157; Cordon-Cardo, 1991 #1182; Davies, 1996 #197; Farinas, 1998 #388; Glass, 1991 #1181; Huang, 1999 #149; Kaplan, 1991 #1183; Klein, 1991 #1184; Lamballe, 1991 #1180; Soppet, 1991 #1173; Squinto, 1991 #1172]. Additionally, p75 can be independently activated by each neurotrophin, or may partner with any one of the neurotrophin trk receptors to mediate specific responses [Chen, 2009 #2014].

NT-3 roles in peripheral sensory and sympathetic nervous systems and tissues

Similar to other neurotrophins, NT-3 associated with the peripheral nervous system is essential for the survival of a large proportion of sensory and sympathetic neurons. In the initial characterizations, the survival effects of each neurotrophin were found to be associated with neurons mediating a different somatosensory modality and thus were described as "*modality specific*" [Farinas, 1999 #647; Snider, 1994 #788]. For example,

NT-3 supports large-diameter, parvalbumin-immunopositive dorsal root ganglion (DRG) neurons that convey proprioceptive signals [Ernfors, 1994b #599; Farinas, 1994 #227; Tessarollo, 1994 #220], whereas nerve growth factor is essential for survival of small-diameter DRG neurons that transmit pain signals [Crowley, 1994 #1205; Smeyne, 1994 #1207; Stucky, 1999 #1043].

NT-3 acts sequentially to support sensory and sympathetic neuron survival, initially during gangliogenesis, subsequently during axon growth toward the target tissue, and then after target innervation [Farinas, 1996 #187; Francis, 1999 #1796; Kuruvilla, 2004 #1794; Patapoutian, 1999 #153; White, 1996 #1751; Zhou, 1996 #637]. For some pathways neurotrophin or nerve growth factor requirements may switch upon target innervation [Buchman, 1993 #235; Ernfors, 2001 #1750].

Several actions of NT-3 in addition to its effects on neuron survival have been characterized in the sensory and sympathetic nervous systems, including roles in neuronal differentiation [Ernfors, 2001 #1750], axon growth [Genc, 2004 #1755; Tucker, 2001 #1746], and nerve terminal formation, including size and structure [Lentz, 1999 #155; Ulupinar, 2000 #652], degree of contact with their accessory cells, and survival of their accessory cells [Albers, 1996 #192], receptor maintenance [Airaksinen, 1996 #204] and neurotransmission [Arvanov, 2000 #1822; Oestreicher, 2000 #748]. Further, NT-3 produced in peripheral tissues may have effects on non-neural tissues as has been observed in hair follicles where it stimulates growth during development and inhibits it in adulthood [Botchkarev, 2004 #1752]. Finally, exogenous NT-3 can affect sensory nerve activity [Mizisin, 1999 #142] as well as GI transit and myoelectric activity [Chai, 2003 #1754; Coulie, 2000 #1753; Parkman, 2003 #1757].

NT-3 Roles in vagal sensory innervation of the GI tract

NT-3, acting in part through activation of trkC, is essential for the survival of a large proportion of vagal sensory neurons: NT-3 and trkC homozygous mutants have 34-47 and 14% loss of neurons from the nodose-petrosal ganglion complex, respectively [Ernfors, 1994b #599; Farinas, 1994 #227; Liebl, 1997 #170; Tessarollo, 1997 #169]. Also, NT-3 supports survival of a different population of vagal sensory neurons than those supported by the other neurotrophins [ElShamy, 1997 #174]. However, in contrast to *modality-specific* regulation of somatosensory neuron survival by neurotrophins, their regulation of vagal sensory neuron survival may follow an "*organ-specific*" principle with each neurotrophin regulating survival of all the sensory receptor types that innervate an organ system [Brady, 1999 #384]. Consistent with this principle, different neurotrophins support survival of sensory innervation of different GI organs. Vagal sensory innervation of the esophagus appears to be dependent on NT-3 as the low threshold slowly adapting intraganglionic laminar ending (IGLE)-type mechanoreceptors that predominate in the muscle wall are reduced by 65 and 40% in NT-3 and trkC heterozygous mutants, respectively [Raab, 2003 #1678]. In contrast, the vagal sensory innervation of the small intestine appears to be dependent on NT-4 as homozygous NT-4 mutants have almost complete loss of IGLEs from the small intestine, but normal IGLE density and structure in the stomach [Fox, 2001b #791].

The source of NT-3 regulating development of vagal sensory innervation of the GI tract

The effects of NT-3 on survival of vagal sensory neurons reviewed above and other potential effects of NT-3 on vagal afferent development could be mediated by its expression in the region of the developing nodose ganglion [Ernfors, 1992 #627], or by NT-3 present in embryonic and postnatal GI tract tissues innervated by vagal sensory neurons, similar to what has been observed in other sensory systems as described above. Indeed, NT-3 expression in the embryonic GI tract has been observed in the rat stomach [Scarisbrick, 1993 #1747], the mesenchyme of the avian gut [Chalazonitis, 1996 #205; Le Douarin, 1999 #1748], at the mesenchyme-epithelium boundary in the upper esophagus of the mouse [Patapoutian, 1999 #153], and in the postnatal mouse colon [Lommatzsch, 2005 #1756]. However, for the mouse, only the upper esophagus and colon have been examined, and in all of these species and organs that have been investigated the details of the locations and timing of NT-3 expression are lacking.

It is highly probable that this NT-3 expression within GI tract tissues is involved in mediating NT-3 effects on development of vagal sensory innervation of the GI tract. Consistent with this hypothesis, trkC as well as trkB, which can also be activated by NT-3 (see above) are expressed by nodose ganglion neurons from E13-E18 [Huber, 2000 #1290; Huang, 1999 #149; Ernfors, 1992 #627], and NT-3 undergoes retrograde transport in vagal sensory axons, albeit this has only been demonstrated in adult rats [Helke, 1998 #167]. These findings are significant because neurotrophin effects on neuronal survival are thought to be dependent on retrograde transport of the internalized neurotrophin-trk receptor complex to the cell body (e.g., [Grimes, 1997 #1202; Grimes, 1996 #1203]; but see [MacInnis, 2002 #1204], although some NT-3 actions may not require this process [Kuruvilla, 2004 #1794]. Similarly, NT-3 expression

in the GI tract wall has been suggested to mediate survival and differentiation of a subset of enteric neuron innervation of the intestine (Chalazonitis, 2001). Consistent with these NT-3 roles, developing myenteric neurons express trk receptors [Lamballe, 1994 #645; Sternini, 1996 #807], and adult enteric neurons exhibited retrograde transport of radiolabeled NT-3 injected into their target tissues, the mucosa and the muscle wall, suggesting they may normally obtain NT-3 in this manner. Moreover, myenteric neuron density was increased or decreased with overexpression or knockout of NT-3, respectively, and submucosal plexus neurons also exhibited reduced density in NT-3 deficient mice.

Investigation of the roles NT-3 produced by peripheral tissues plays in development of vagal sensory innervation of the GI tract, or for that matter, its roles in development of other extrinsic and intrinsic innervation of the GI tract, requires a much more detailed understanding of the spatiotemporal pattern of NT-3 expression in the developing GI tract than is currently available. Knowledge of which tissues express NT-3 and receive vagal sensory innervation would identify the vagal sensory pathways likely to be regulated by NT-3, and determination of when this expression occurred would distinguish the stage(s) of development at which NT-3 might regulate them (e.g., axon arrival vs. receptor differentiation). Therefore, our aims were to identify which tissues/cell types within the GI tract express NT-3, and to characterize the time course of this expression at ages when vagal axons grow into the GI tract and their nerve terminals begin to differentiate. These findings will provide a basis for formulating and testing hypotheses about the roles of peripheral NT-3 expression in GI vagal afferent development, including the *organ-specific* principle of neurotrophin action.

MATERIALS AND METHODS

Subjects

The NT-3^{LacZ} mouse [Farinas, 1994 #227] obtained on an ICR out bred background was backcrossed up to 11 generations with C57BL/6 mice obtained from Harlan Industries (Indianapolis, IN) and bred in our lab. Mice were maintained at 23ºC on a 14:10 hr light:dark schedule, with ad libitum access to tap water and Laboratory Rodent Diet 5001 (PMI Nutrition International. St. Louis, MO). All procedures were conducted in accordance with Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985) and American Association for Accreditation of Laboratory Animal Care guidelines and were approved by the Purdue University Animal Care and Use Committee.

Rationale for using NT-3^{LacZ} mice. Mice that had the *LacZ* reporter gene "knocked into" the NT-3 locus (NT-3^{LacZ} mice; [Farinas, 1994 #227], and thus expressed β-galactosidase under control of the NT-3 promoter were utilized to characterize NT-3 expression in the developing GI tract. The rationale for using NT-3^{LacZ} mice in such studies has been discussed extensively (e.g., [Farinas, 1996 #187; Vigers, 2000 #1685; Vigers, 2003 #1682]. Of particular relevance, NT-3^{LacZ} mice have been employed in numerous studies of detailed NT-3 expression patterns in many different organs and tissues [Bennett, 1999 #1825; Farinas, 1996 #187; Farinas, 2001 #1826; Hess, 2007 #1828; Tojo, 1995 #1824; Vigers, 2000 #1685; Vigers, 2003 #1682; Wilkinson, 1996 #188; Yee, 2003 #1359]. Moreover, these mice have yielded higher resolution cellular

expression patterns than had been achieved with *in situ* hybridization (e.g., somatosensory system; [Farinas, 1994 #227; Jones, 1994 #414]. Also, the patterns of NT-3 expression obtained in these mice accurately reproduced the patterns derived using methods that reveal endogenous expression (Farinas et al, 1996).

Tissue preparation

Matings were set up between heterozygous NT-3^{LacZ} mice, or between heterozygous NT-3^{LacZ} mice and wild-type mice. Noon of the day a copulatory plug was observed was designated E0.5 and the day of birth as postnatal day (P)0. Embryos were harvested on E12.5, E13.5, E15.5 and E17.5, and GI tracts were dissected from P4 mice. Results were based on staining patterns in 3 or more independent litters at each age studied, and multiple embryos or neonates from each litter were examined. Homozygous and heterozygous mutants and wild types were genotyped by PCR using DNA extracted from a tail sample for mice, or the yolk sac for embryos (PCR primer sequences: wild-type allele - forward: ACT ACG GCA ACA GAG ACG CTA C, reverse: ACA GGC TCT CAC TGT CAC ACA C; mutant allele - forward: GTG CCA GCG GGG CTG CTA AAG CGC, reverse: CTG CAT TCT AGT TGT GGT TTG TCC AAA CTC ATC).

Embryos. Pregnant mice were euthanized by cervical dislocation, and then the uterus was removed and placed in chilled phosphate buffered saline (PBS) on ice. Each embryo was dissected free of their deciduum and yolk sac, and then the skin and muscle enclosing the abdomen were peeled back to expose the abdominal organs. Embryos were then fixed for 30 min on ice with1% paraformaldehyde, 0.02%

gluteraldehyde, 0.5 mM EGTA, and 2 mM $MgCl_2$ in 0.1M sodium phosphate buffer, pH 7.4.

Postnatal mice. Neonatal mice were deeply anesthetized with a lethal dose of methohexital sodium (Brevital Sodium, King Pharmaceuticals, Inc., Bristol, TN; 100 mg/kg i.p.). When mice were unresponsive to nociceptive stimuli, the abdomen and thorax were exposed and animals were perfused transcardially at a flow rate of 3 ml/minute with 0.9% saline for 5 min at RT followed by the same fixative used for embryos for 30 min at 4ºC.

Histochemical staining of β-galactosidase

Immediately after fixation the embryos or abdominal organs were stained with X-gal as previously described [Fox, 2000 #333]. After staining was completed, tissue was postfixed 48 hr in 4% paraformaldehyde at 4ºC, washed with PBS and then transferred to 10% buffered formalin at 4ºC for a minimum of 5 days. Then the tissues were embedded in paraffin, sectioned at 8 μm thickness, air dried on gelatin-coated slides, alternate ribbons of sections were counterstained with 0.1% neutral red, and all sections were dehydrated in a series of graded alcohols (70%, 95%, 2 x 100%; 2 min each), cleared in xylene (3 x 2 min) and coverslipped with Cytoseal (Richard Alan Scientific, Kalamazoo, Michigan). Additionally, IHC detection of β-galactosidase in the E17.5 gut using the methods described below was employed to validate the results obtained by X-gal staining.

Antibody characterization

Information on primary antibodies used in mouse embryonic tissues, including immunogens, is summarized in Table 1. Additional information on controls for specificity of each of these antibodies is provided below.

The β-galactosidase antibody employed was produced in guinea pigs and its specificity characterized by Yee et al. [Yee, 2003 #1359]. No immunoreactivity was detected after applying the antibody to mouse lingual tissue that did not contain β-galactosidase. Moreover, application of this antibody to tissues from several LacZ transgenic strains with tissues expressing β-galactosidase resulted in strong immunoreactivity. Additionally, when the β-galactosidase immune sera were preadsorbed with β-galactosidase, no immunoreactivity was detectable after its application to β-galactosidase - expressing mouse lingual tissue. In the present study, this antibody also showed complete correspondence with X-gal staining of β-galactosidase expression in developing gastric and intestinal smooth muscle, esophageal epithelium and gastric lamina propria.

Goat polyclonal anti-c-Kit (lot numbers H2907, C2108) was raised against a peptide from the carboxy terminus of mouse c-Kit. The molecular weight of precursor c-Kit is 120 kDa and the mature form is 145 kDa. In Western blots of lysates (some immunoprecipitated) from several cell lines (manufacturer technical information; [Jahn, 2002 #1984] and cultured fetal cells [Nobuhisa, 2003 #1983] stained both the precursor and mature bands. Moreover, immunostaining using this antibody in tissue sections was prevented by pre-incubation of the antibody with c-Kit blocking peptide [Schrans-Stassen, 1999 #1985]. Additionally, this antibody has been used to label cells in the GI tract wall of the mouse, rat and guinea pig with distributions and morphologies

consistent with those of the several ICC classes [Fox, 2000 #689; Ho, 2003 #1986; Pham, 2002 #1982].

The mouse IgG2b neuronal protein HuC/HuD (HuC/D) monoclonal (clone 16A11) antibody (lot number 42804A) recognizes an epitope within the carboxy terminal domain of HuD, resulting in labeling of neuronal cell bodies and nuclei [Marusich, 1994 #1964]. This antibody recognizes neuronal proteins of the Elav family, including HuC and HuD, which are RNA-binding proteins, and Hel-N1. The presence of Hu proteins acts as a marker for both newly committed postmitotic neurons and mature neurons [Barami, 1995 #1961; Marusich, 1994 #1964; Young, 2005 #1962]. The staining pattern of cellular morphology and distribution observed in the enteric nervous system in the present study was the same as previously described [Lin, 2002 #1966; Murphy, 2007 #1965; Young, 2005 #1962]. Importantly, the staining pattern of biotin-conjugated HuC/D monoclonal antibody was identical to that of the unconjugated antibody [Hoff, 2008 #1963].

The polyclonal rabbit antibody directed against p75 (lot number 606031680), the low affinity neurotrophin receptor, was raised against the extracellular fragment specified by exon 3 sequences of the murine p75 gene (manufacturer's technical information). Specificity of this antibody was originally characterized by Huber and Chao [Huber, 1995 #1976] and in Western blots it labeled a single band of about 75 kDa in brain [Huber, 1995 #1976], embryonic testis [Campagnolo, 2001 #1974], and importantly, GI myenteric plexus [Lin, 2005 #1977]. In the present study the morphology and distribution of immunoreactivity produced using anti-p75 was consistent with glial and

neural precursors, and with staining patterns produced by the same antibody in similar preparations [Lin, 2005 #1977; Vannucchi, 2000 #1971; Yan, 2008 #1970].

A polyclonal rabbit antibody directed against the established glial marker S100 (lot number 15317) was employed, which stains the cytoplasm, labeling glial somata and processes. Specificity was demonstrated by two-dimensional immunoelectrophoresis, which identified one distinct double peak with human and cow brain extracts corresponding to S100a and –b and found no reaction with human plasma or cow serum (manufacturer's technical information). Further, it was found using an indirect ELISA that anti-S100 did not react with human plasma or cow serum.

IHC detection of sites of NT-3 expression (β-galactosidase protein)

IHC co-localization of an antibody that detects *LacZ* expression (β-galactosidase) with an antibody selective for either neurons (anti-HuC/D), glia (anti-s100), undifferentiated neural crest precursors (anti-p75), or interstitial cells of Cajal (ICCs; anti-c-Kit) in heterozygous NT-3lacZ embryos was utilized to determine whether NT-3 expression occurred in these cell types. Tissue sections from wild-types were stained in parallel to examine the specificity of the β-galactosidase antibody. Embryos harvested at E17.5 as described above were immersion fixed in 4% paraformaldehyde at 4^{o}C for 24 hr, rinsed in PBS, incubated in 15% sucrose PBS for 1 hr at 4^{o}C, followed by 30% sucrose overnight (ON) at 4^{o}C, then a 1:1 mixture of 30% sucrose and OCT compound (Tissue-Tek, Miles Inc) ON at 4^{o}C, and frozen in 100% OCT using liquid nitrogen. Sections were cut at 10 μm thickness and air dried on gelatin-coated slides. Additionally, a small number of wild-type embryos were harvested at E14.5 for

evaluation of NT-3-LIR at an earlier stage of expression (see Results) and were treated the same as described above for E17.5 embryos.

IHC procedures. All steps were done at RT unless indicated. Tissue sections were washed in PBS, incubated 1 hr in blocking solution (10% normal donkey serum, 0.5% triton X-100, 2% BSA, and 0.1% sodium azide), and then incubated ON in the first primary antibody at 4^{o}C. These, as well as all other primary and secondary antibodies employed in the present experiments were diluted with 1% BSA, 2% normal donkey serum, 0.3% Triton X-100, and 0.1% sodium azide. After washing with PBS, sections were incubated in the first secondary antibody for 2 hr and then washed again with PBS. Next, the sections were incubated for 1 hr in blocking solution and then in the second primary antibody ON at 4^{o}C. Sections were washed in PBS and then incubated in the second secondary antibody for 2 hr. After washing in PBS sections were mounted in glycerol, coverslipped and sealed with nail polish. The secondary antibodies used included donkey rhodamine red X (RRX) - conjugated anti-goat or anti-guinea pig IgG (H+L) and donkey fluorescein iso-thiocyanate (FITC) – conjugated anti-rabbit, anti-goat or anti-guinea pig IgG (H+L; Jackson ImmunoResearch Laboratories, Inc.). The mouse monoclonal HuC/D antibody employed was tagged with biotin, which avoided use of an anti-mouse secondary and the associated high background. To label this antibody streptavidin tagged with tetramethyl rhodamine iso-thiocyanate (TRITC) or FITC was applied in the same manner as described for secondary antibodies. To control for antibody interactions, the majority of staining runs for each pair of primary antibodies included several sections that were taken through the entire double-staining protocol, omitting the first primary antibody on some sections and the second primary

on other sections (incubation in primary antibody diluent alone was substituted). Further, to control for non-specific staining by secondary antibodies, staining produced by each primary antibody alone was compared with staining following omission of that primary antibody (incubation in primary antibody diluent alone was substituted) with all other protocol steps kept constant. **[Emphasize the outcomes of these control expts in the Results section]**

Microscopy and Photomicrography

X-gal-stained tissue was examined with standard bright-field or differential interference contrast illumination and rhodamine and fluorescein fluorescence was visualized using standard filter sets (Leica DM5000 microscope; fluorescence filter cubes L5 and Y3, respectively). Photomicrographs were acquired directly with a video camera (Spot RT Slider; Diagnostic Instruments, Inc., Sterling Heights, MI). The fluorescence-stained tissues were also scanned with an Olympus BX-DSU spinning disk confocal microscope operated using Slidebook software (v.4.1, Intelligent Imaging Innovations, USA). Image processing, three-dimensional reconstructions and two-dimensional projections of z-series stacks of optical sections were also performed using the Slidebook software. Standard fluorescence microscopy was utilized to identify regions of tissue sections with potential for co-localization of two different primary antibodies based on the apparent overlap of their respective rhodamine or fluorescein labels. Regions to be imaged were randomly selected and then a series of confocal scanning optical sections through the z-axis of each region were collected. The series of sections collected at each site was examined to determine whether both labels were

present in the same tissue elements. Three criteria were employed to determine co-localization: (1) In composite images of the FITC and TRITC or Texas Red channels, elements exhibited a yellow pseudocolor as a result of the combined green pseudocolored FITC channel and red pseudocolored TRITC or Texas Red channel, (2) The separate green- and red-labeled elements were in focus as determined by scanning through the z-series, and (3) The shapes of the green- and red-labeled elements exhibited partial or complete matching of in focus tissue elements. Photoshop software (version 6.0 Adobe Systems, Mountain View, CA) was used to a) apply scale bars and text, b) adjust brightness and contrast, c) apply color correction, and d) organize the final layouts for printing.

RESULTS

β-galactosidase expression was localized using X-gal staining of the GI tract of embryonic and neonatal NT-3^{lacZ} mice, which express β-galactosidase under control of the NT-3 promoter. Additionally, IHC detection of β-galactosidase in these mice at E17.5 was used to validate the findings obtained with X-gal staining. This age was chosen because *LacZ* expression levels appeared to be high in most tissues and all tissues had developed sufficiently to be clearly distinguished. β-galactosidase expression was observed in embryonic tissues previously reported to express NT-3, including the ventral motor neurons within spinal gray matter and hair follicles in the skin, suggesting accurate regulation of expression has been maintained in our line of NT-3^{lacZ} mice (not shown; [Botchkarev, 2004 #1752; Buchman, 1993 #235; Ernfors, 1991 #1941]. High levels of X-gal staining were apparent in wholemounts at one or

more of the ages examined (E12.5, E13.5, E15.5, E17.5 and P4) in all the major GI organs studied including the esophagus, stomach, pylorus, small intestine, cecum and large intestine. There was no stain in the GI tracts of wild-type embryos at ages E12.5-E15.5, but staining was present in the mucosa at many levels of the GI tract at E17.5 and P4, suggesting staining of this tissue in E 17.5 and P4 transgenic specimens was artifactual. Artifactual X-gal staining due to endogenous enzymes with similar activity to the bacterial galactosidase has been reported in several tissues, including the adult mammalian intestinal mucosa [Coates, 2001 #1948; Lojda, 1974 #1946; Sanchez-Ramos, 2000 #1947; Weiss, 1999 #1945]. Consistent with this interpretation, the pattern of NT-3 expression (X-gal staining) in NT-3^{lacZ} embryos as detailed below, was replicated in all tissues at E17.5 by β-galactosidase-LIR except for the mucosa of the stomach and intestines (Figs. 1E and 2A-C). This method also failed to label the mucosa of these organs in wild-type embryos. An additional implication of these findings using IHC detection of β-galactosidase is that masking of actual mucosal NT-3 expression by the artifactual X-gal staining of this tissue was unlikely.

The results in Table 2 summarize localization of non-artifactual β-galactosidase expression at the 5 ages studied in detail, E12.5, E13.5, E15.5, E17.5 and P4.

NT-3 expression in the esophagus. **[Make clear here or in Fig Legend that the esoph images are different ages, not just different levels]** No NT-3 expression was detected in the esophagus at E12.5 or E13.5. Moderate expression was first observed at E15.5 and was restricted to the epithelium (Fig. 1A). At E17.5 epithelial NT-3 expression was present throughout most of the length of the esophagus (Fig. 1B), but it increased from moderate levels in the region of the LES to high levels in more anterior

regions of the esophagus, whereas most of this expression at P4 was at high levels (Fig. 1C). Additionally, NT-3 expression in the circular muscle of forestomach (see below) extended into the region of the developing LES (Fig. 1D). No other esophageal tissues exhibited NT-3 expression at any of the ages examined.

NT-3 expression in the stomach. The wall of the stomach in E12.5 embryos consisted of a thick layer of mesenchyme surrounding the single-cell thick epithelial layer (Fig. 3A,B). NT-3 expression was present in the entire thickness of this mesenchyme, but was stronger in the anterior portion of the caudal stomach region that develops into the corpus and antrum. In contrast there was no expression in the epithelium. A similar expression pattern was observed at E13.5, although in some specimens the tissue layers of the stomach wall had begun to differentiate and NT-3 was expressed in those layers that develop into the lamina propria and circular and longitudinal smooth muscle (Fig. 3C,D). By E15.5 all of the layers of the stomach wall had become distinct in all specimens. Strong NT-3 expression at this age was restricted to the longitudinal and circular smooth muscle layers with the strongest expression in the antrum occurring on its anterior side (Fig. 3E,F and 4A,B). Additionally, expression in the forestomach was restricted to the circular layer of the muscle wall. Expression was also present in the lamina propria of the mucosa of the antrum and corpus, but it was consistently weaker than in the muscle layers. This overall expression pattern in the stomach wall was maintained at E17.5 and P4 (Figs. 4C,D and 5A-E) except that expression was slightly increased in the lamina propria on E17.5, and appeared to decline slightly in the muscle layers postnatally. There was no clear expression in the submucosa, epithelium, or enteric plexuses at any of the ages examined.

NT-3 expression in the pylorus. The spatial and temporal patterns of NT-3 expression in the pylorus largely paralleled that of the adjacent caudal portion of the stomach. At E12.5 and E13.5 NT-3 expression was already present in the mesenchyme of the pylorus and it was stronger in the anterior portion of the wall. Interestingly, this strong expression extended into the ventral mesogastrium (mesenchyme) immediately external to the pylorus and stretched short distances in the rostral direction (adjacent to the caudal stomach) and in the caudal direction (alongside the proximal duodenum). At later stages (E15.5 and E17.5) strong expression became concentrated in the external smooth muscle layers (Fig. 4A-D). In particular, the expression levels were strong through the entire width of the pyloric muscle wall, which was predominantly a thick circular muscle layer. Postnatally (P4) this tissue began to show a decline in expression level. Additionally, the weak expression that occurred in the lamina propria of the antrum extended into the pylorus and exhibited a similar time course. The only tissues that did not exhibit clear expression at any of the ages studied were the epithelium, submucosa and enteric plexuses.

NT-3 expression in the small intestine. The NT-3 expression present throughout the thickness of the muscle wall in the caudal stomach and pylorus became restricted to the circular smooth muscle layer in the duodenum – an expression pattern that was dominant for the muscle wall in all the compartments of the small intestine. Expression in the small intestine muscle was first observed at E12.5, and at this age and E13.5 it was present in the mesenchyme, or developing external muscle layers where it occurred predominantly in the circular muscle layer (Fig. 6). By E15.5 this expression was restricted to the circular muscle layer (Fig. 7A,B), and it remained so at E17.5 and

P4, although expression levels began to decline by P4 (Figs. 7C,D and 8). The tissues that did not exhibit expression at any of the ages examined included the mucosa, submucosa, and enteric plexuses.

NT-3 expression in the cecum. The expression pattern that dominated in the cecum was similar to the small intestine. In most specimens NT-3 expression was observed only in the circular layer of the muscle wall and this was stronger in the body of the cecum near its junctions with the large and small intestines and gradually became weaker and typically disappeared toward the apex of the cecum. In some specimens the expression pattern near the junction region of the cecum and large intestine was similar to that of the colon (see below) – expression occurred in both smooth muscle layers, but was typically weak in the longitudinal layer. This overall expression pattern was observed at E15.5 (Fig. 9A,B), E17.5 (Fig. 9C,D) and P4. At E12.5 and 13.5 the cecum was difficult to identify unequivocally in sections. The tissues that did not exhibit expression at any of the ages examined included the mucosa, submucosa, enteric plexuses and most of the longitudinal muscle layer.

NT-3 expression in the large intestine. In contrast to the pattern of NT-3 expression observed in the small intestinal muscle layers, at all ages examined, the pattern of labeling in the large intestinal muscle layers was more similar to that observed in the stomach. At early ages, E12.5 and some E13.5 specimens, the muscle layers were not differentiated and this NT-3 expression appeared diffuse throughout the mesenchyme (Figs. 6A-D and 10A,B), whereas in other E13.5 embryos it was mostly restricted to both layers of the forming muscle wall (Fig 10C-F). By E15.5 and at later stages, expression was present at high levels in the circular muscle and varied from

undetectable to high levels in the longitudinal smooth muscle layer (Figs. 11-13). The tissues that did not exhibit expression at any of the ages examined included the mucosa, submucosa, and enteric plexuses.

NT-3 expression in tissues associated with abdominal GI organs. The smooth muscle of some mesenteric blood vessels exhibited NT-3 expression by E15.5 (Fig. 14A,B) and by E17.5 (Figs. 9C,D and 14C,D) and P4 expression was also present in the smooth muscle of blood vessels that supplied the stomach (Figs. 2A and 5B,C) and liver, those that exited the mesentery to supply the intestines (Fig. 8), as well as those within one of the liver lobes adjacent to the stomach. Interestingly, there was also NT-3 expression in a thin layer at the edge of the liver lobe that was immediately anterior to the stomach/pylorus and this was observed at all ages examined (e.g., Fig. 3A,B). Moreover, at early stages this portion of the liver was continuous with ventral mesogastrium adjacent to the caudal stomach, which also exhibited NT-3 expression (see above).

NT-3 expression in enteric nerve plexuses. Based on examination of the X-gal-stained tissues described above, there were no indications of significant NT-3 expression within the neurons and glial cells associated with the submucosal plexus in any of the organs studied. Moreover, there were no patterns of staining within the muscle walls of the organs examined suggestive of myenteric ganglion structure. Nevertheless, it is possible that small numbers of labeled cells in the myenteric plexus were obscured by the intense expression in the immediately adjacent smooth muscle layers. This possibility was examined by employing IHC double-labeling to determine whether markers for neurons (anti-HuC/D), or glial cells (anti-S100), or their precursors

(anti-p75) were associated with NT-3 expression (anti-β-galactosidase). No evidence was found for NT-3 expression in any of these cell types in the myenteric plexus as clear instances of double-labeled cells were not observed (examples: Fig. 15). These data are summarized in the top half of Table 3.

DISCUSSION

Spatiotemporal pattern of NT-3 expression

Each GI organ studied exhibited NT-3 expression at one or more of the ages examined. The spatial pattern of NT-3 expression was characterized by two principles. First, NT-3 expression in the majority of the GI tract organs, including the stomach, small intestine, cecum, and large intestine was smooth-muscle specific: it was largely restricted smooth muscle cells comprising the outer layers of the developing stomach, cecum and intestines and the walls of blood vessels that supply the GI tract. The one exception to this principle was in the lamina propria of the stomach mucosa, although expression levels in this tissue appeared to be lower than in the smooth muscle. Second, each organ investigated exhibited a unique expression pattern that differed from others in terms of which muscle layer exhibited NT-3 expression and whether any additional tissues exhibited expression. For example, although NT-3 was expressed in the circular muscle layer of the stomach, intestines and cecum, expression in the longitudinal muscle layer only occurred in some regions of the stomach and colon, thus distinguishing the expression patterns in these organs from those of the small intestine and cecum. Further, the lamina propria of the mucosa only exhibited expression in the

stomach, which distinguished the stomach pattern from that of the colon. Also, NT-3 expression in the esophagus exhibited a dramatically different pattern from all the other GI organs, being restricted to the mucosal epithelium. Although there appeared to be some differences in the temporal pattern of NT-3 expression across and within tissues, these observations must be interpreted cautiously because the persistence of β-galactosidase protein in the various stained tissues is not known (its half-life in neurons is about eight hours; [Pham, 1999 #1823]. NT-3 expression in the mesenchyme of the GI wall was present at the earliest age examined, E12.5, and started to become distinct in tissue layers developing into the smooth muscle and lamina propria at E13.5. In contrast, NT-3 expression in vascular smooth muscle and the esophageal epithelium were first observed at E15.5. Moreover, at E12.5 expression in outer gut wall smooth muscle already appeared to be at high levels, whereas initially, at E15.5 expression in the vascular smooth muscle and esophageal epithelium was at low levels that increased gradually with age. Additionally, NT-3 expression was maintained in most of these tissues at least until P4, although levels in some had begun to decline.

In general, the present characterization of embryonic and neonatal NT-3 expression correlates well with the results of previous studies that examined NT-3 expression in the alimentary tract of embryonic tissues of mice, rats and birds [Chalazonitis, 1996 #205; Le Douarin, 1999 #1748; Patapoutian, 1999 #153; Scarisbrick, 1993 #1253]. **[This next statement makes it sound like you discovered little new information so change it please. Maybe spell out more clearly what these studies found and compare what we found above]** The major difference between our results and previous studies was that we identified low expression levels in a tissue that had not previously been reported

to express NT-3 at embryonic ages, the mucosa of the stomach antrum and corpus. Additionally, we observed moderate to strong NT-3 expression levels in the walls of blood vessels supplying the GI tract. To our knowledge this is the first demonstration of such expression. Previously, NT-3 expression had been observed in smooth muscle cells of blood vessels of the cardiovascular system, major abdominal vessels [Scarisbrick, 1993 #1253], and vessels associated with the stellate and superior cervical ganglia and their target organs [Francis, 1999 #1796]. The sensitivity of the X-gal stain combined with greater cellular resolution as compared with *in situ* hybridization may have permitted visualization of sites such as the lamina propria where expression levels were lower than in mesenchyme or smooth muscle.

Since a subpopulation of myenteric neurons is dependent on NT-3 for differentiation and survival [Chalazonitis, 2001 #979] and small numbers of myenteric neurons have been observed to contain NT-3 in the mature GI tract of the rat [De Giorgio, 2000 #806], we investigated whether these neurons express NT-3 during development. However, the region of the GI tract wall that contains the myenteric plexus, which is situated between the longitudinal and circular smooth muscle layers, did not exhibit X-gal staining or β-galactosidase immunolabeling in NT-3^{LacZ} mice at any of the ages examined. These observations suggest none of the myenteric plexus elements express NT-3, including myenteric neurons, glial cells, undifferentiated neural crest cells and ICC-MYs. Consistent with this interpretation, at E17.5 immunolabeling for β-galactosidase did not co-localize with markers for any of these myenteric elements (HuC/D, S100, p75 and c-Kit, respectively).

Implications of spatiotemporal pattern of NT-3 expression

One interesting aspect of the different spatial patterns of NT-3 expression across GI organs was that some of the organs, or their compartments, including the small intestine and forestomach exhibited expession in the circular muscle layer, but not the longitudinal layer, whereas the remaining ones showed expression in both layers – a pattern that was highly consistent across specimens and ages. There are developmental, structural and functional differences between the longitudinal and circular muscle layers that could be causally related to this expression pattern [El-Yazbi, 2007 #1800; Shuba, 2006 #1798; Spencer, 2006 #1799; Takahashi, 1998 #1944]. One particularly intriguing difference involves the localization of precursors of the classes of interstitial cells of Cajal (ICCs) that depend on c-Kit and steel factor for proliferation during development. These include the intramuscular class (ICC-IM) of the forestomach, and the ICC-MY of the small intestine [Burns, 1996 #441; Fox, 2002 #964; Huizinga, 1995 #499; Ward, 1994 #445]. In the small intestine these classes of ICCs arise from mesoderm-derived precursor cells in the longitudinal muscle layer that initially express c-Kit and smooth muscle myosin heavy chain (smmhc) and later, between E14.5 and E18.5, either downregulate smmhc and become ICCs or downregulate c-Kit and become smooth muscle cells [Kluppel, 1998 #490; Torihashi, 1997 #439]. Initially, c-Kit is also expressed in the circular muscle, but it is downregulated before precursors in the longitudinal layer begin to diverge toward ICC or smooth muscle fates. This raises the possibility that NT-3, or a co-expressed gene suppresses c-Kit expression in circular smooth muscle precursor cells, resulting in loss of their potential to differentiate into ICCs.

The most dramatic difference in the patterns of NT-3 expression was between the esophagus and the more distal GI organs, being restricted to the epithelium rather than to the external smooth muscle layers. This localization is consistent with previous observation of NT-3 expression at the mesenchyme-epithelium boundary in the upper esophagus at E16.5 [Patapoutian, 1999 #153]. This difference in NT-3 expression patterns suggests the genetic program that regulates esophagus development is largely distinct from those for the more distal GI organs. This possibility is consistent with additional differences in esophageal development as compared with the other GI organs. For example, a greater proportion of the neural crest cells that migrate to the esophagus originate from the trunk as opposed to the hindbrain [Durbec, 1996 #1943] and only the esophageal smooth muscle transdifferentiates into striated muscle [Kablar, 2000 #1819; Patapoutian, 1995 #1820]; but see [Rishniw, 2003 #1818]. The role of NT-3 in the esophageal epithelium may be in the survival or development of its innervation, or possibly in the survival or renewal of epithelial cells, which exhibit frequent turnover.

The lack of NT-3 expression in the muscle wall of the abdominal and thoracic esophagus between E13 and P4 is consistent with earlier evidence suggesting loss of vagal sensory neurons in NT-3-deficient mice, which would include the loss of IGLEs in these mice [Raab, 2003 #1678], is probably due to the loss of NT-3 from the region of the nodose ganglion, rather than to loss from the target organ [ElShamy, 1997 #174]. The lack of NT-3 expression in the outer smooth muscle layers of the esophagus further implies that once the axon terminals of IGLE vagal mechanoreceptors reach the esophagus during development, which begins around E15 [Sang, 1998 #810], their survival and differentiation is supported by factors other that NT-3.

[rewrite this section considering tissues that have trk receptors, and therefore if they took up NT-3 from muscle, what might happen *GI tract tissues and cell types that sequester and potentially utilize secreted NT-3*

Second, one reason for the persistence of NT-3 in vascular smooth muscle cells or any other cell type expressing NT-3 would be if it had autocrine function, and conversely, its absence would be associated with a lack of autocrine function. Consistent with this possibility, trk receptors, including trkC are present in vascular smooth muscle cells and appear to mediate autocrine functions that are known to be regulated by neurotrophins, including cell migration and survival [Donovan, 1995 #1830]. Conversely, as trk receptors have not been observed in smooth muscle cells of the GI wall (e.g., [Lamballe, 1994 #645; Sternini, 1996 #807], it is more likely that NT-3 would have a paracrine role rather than be sequestered or utilized by these cells.

In addition to the possible autocrine effects of NT-3 produced by vascular smooth muscle cells described above, it has been shown to have paracrine effects, providing an intermediate target signal that promotes the growth or guidance of sympathetic axons growing in close proximity to blood vessels by activating trkA receptors on those axons, enabling them to reach their final target [Francis, 1999 #1796; Kuruvilla, 2004 #1794]. The potential for NT-3 produced in blood vessel walls to regulate vagal sensory axon development has not been directly examined, but since vagal axons also grow along blood vessels to reach and enter the gut wall, the expression pattern we observed is suggestive.

NT-3-LIR in cell types that did not express NT-3. NT-3 is presumably secreted from

smooth muscle cells associated with the outer GI wall. Consequently, developing vagal and sympathetic extrinsic innervation and myenteric intrinsic innervation of the outer muscle layers of the gut wall would have access to this source of NT-3.

This suggests that a small number of myenteric neurons take up NT-3 secreted from the muscle layers, consistent with the dependence of a subpopulation of myenteric neurons on NT-3 for survival [Chalazonitis, 2001 #979] and with the expression of trk receptors in myenteric neurons and processes from mid-gestation to birth [Lamballe, 1994 #645; Sternini, 1996 #807]. The present results also suggested that vagal terminals that innervate the muscle and myenteric layers of the outer GI wall are another major element that takes up NT-3 secreted from the muscle layers. This pattern is consistent with the localization of trk receptors, including trkC to vagal sensory neurons from mid- to late gestation [Huber, 2000 #1290; Huang, 1999 #149; Ernfors, 1992 #627].

Implications of NT-3 expression pattern for the organ-specific hypothesis

At face value, the pattern of NT-3 expression does not appear to support the organ-specific hypothesis of neurotrophin survival action on sensory neurons. In particular, NT-3 expression was distributed throughout similar tissues in several GI organs. If the function of NT-3 is similar in each of these organs this would clearly be a violation of the principle. However, if NT-3 had different functions in each organ, then it is still possible NT-3 survival function is organ-specific. For example, NT-3 may be involved in survival of vagal afferent innervation of one GI organ, but in axon growth or guidance in another [Genc, 2004 #1755; Niwa, 2002 #1942; Paves, 1997 #1773] and receptor differentiation

in yet another [Ming, 1997 #1771].

Many levels of the vagal sensory system are associated with potential sources of NT-3, including their central targets in the brainstem, the nodose ganglion where their cell bodies are located, the blood vessel walls their axons follow on their way to the gut, and within the gut itself. One way to distinguish the roles of each NT-3 source would be to manipulate a one source at a time *in vivo* and examine the effects on vagal development, for example, by using the cre-lox conditional knockout strategy [Sauer, 1998 #1677]. The present analysis represents a prerequisite for this strategy, which is to identify the cell types that express NT-3 in and near the target organs of vagal GI sensory neurons and the ages at which this expression occurs.

Regulation of the NT-3 expression pattern in the developing GI tract.

Little is known about the regulation of neurotrophin expression in the gut during development. Comparison of NT-3 expression patterns across organs identified differences that must be accounted for by a regulatory scheme. For instance, as summarized earlier in this discussion, each GI organ exhibited a unique pattern of NT-3 expression.

Evidence is beginning to accrue, suggesting that Hox genes play a key role in the gene cascade, or genetic program that regulates the formation of the different GI organs from a relatively uniform tube. Therefore, these Hox genes are candidates for contributing to regulation of organ-specific patterns of NT-3 expression. In compartments of the developing insect cuticle (segments) and the vertebrate hindbrain (rhombomeres), Hox genes exhibit overlapping, nested expression patterns with unique

anterior expression boundaries that coincide with the locations where compartment boundaries later form [Lewis, 1978 #1816; McGinnis, 1992 #1817]. The result is a unique combination of Hox genes being expressed in each compartment, which acts as a code that confers the compartment's identity. The Hox proteins present in each compartment activate expression of numerous genes that produce patterns of cell proliferation and migration that contribute to the formation of the unique structures present with each segment.

More recently, a similar relationship between Hox gene expression patterns and individual GI organs has been reported to occur by approximately E10, presaging the locations where the boundaries between organs form [Kawazoe, 2002 #1815; Pitera, 1999 #1804; Sekimoto, 1998 #1813]. Thus, should these organ-specific Hox gene expression patterns provide a code that confers identity to individual gut segments they could directly or indirectly contribute to the organ-specific NT-3 localization patterns that occurred in the GI tract. Moreover, there is some evidence that these expression patterns are maintained into maturity and determine the different identities of stem cell populations in each mature organ [Yahagi, 2004 #1814].

Also consistent with a role for Hox genes in gut pattern formation, manipulations of Hox expression can alter the developmental trajectory of GI organ development and the location of organ boundaries. For example, ectopic expression of *Hoxc8* in the mouse stomach leads to overgrowth of stomach epithelium and extension of the duodenum to the anterior pylorus [Pollock, 1992 #1992]. Also, ectopic expression of the *IDX1* homeobox gene results in alteration of the midgut-hindgut boundary and failure of the cecum to form [Heller, 1998 #1991].

Another potentially important facet of Hox expression in the developing gut is that the expression of many Hox genes is restricted to the mesoderm, suggesting the Hox code functions primarily in mesenchyme specialization and then leads to regional differentiation of gut subdomains through epithelial-mesenchyme interactions [Kawazoe, 2002 #1815]. This regional differentiation could involve activation of NT-3 expression in mesenchyme and smooth muscle locations we observed. Further, some Hox genes are expressed in distinct mucosal tissues [Pitera, 1999 #1804], and therefore, could control the differential NT-3 expression observed in them. For example, Hoxc4 was the only gene among the Hoxc and Hoxd paralogs examined that was expressed in the endoderm of the esophagus at E12.5, which gives rise to the epithelium [Pitera, 1999 #1804]. Thus, Hoxc4 could potentially activate the NT-3 expression observed in this tissue.

Hox genes continue to be expressed at ages when NT-3 is expressed and therefore could activate and maintain gut NT-3 expression directly. In fact, there is evidence for direct Hox - NT-3 interactions in other systems. For example, Barhl1, a homeobox gene, and NT-3 exhibit similar expression patterns in association with cerebellar granule cell development. Further, knockout of either gene, or of NT-3 receptors has similar deleterious effects on granule cell migration and survival during development [Li, 2004 #1806]. Moreover, the 5' flanking sequences of the NT-3 gene were found to contain at least 2 potential Barhl1 homeodomain binding sites. Similarly, there is evidence that a neurotrophin closely related to NT-3, BDNF, which is also expressed in gut-associated smooth muscle [Fox, 2006 #1760] is regulated by the homeobox genes Pitx3 and Hoxa1 [Martinez-Ceballos, 2005 #1808; Peng, 2007 #1807].

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LITERATURE CITED

FIGURE LEGENDS

Fig. 1. Photomicrographs demonstrating the restriction of NT-3 expression in the wall of the esophagus to the epithelium at E15.5 (A), E17.5 (B) and P4 (C). These cross-sections through the thoracic (A,B) and abdominal (C) esophagus were reacted with X-gal, which resulted in a blue stain in cells expressing β-galactosidase. (D) A longitudinal section through the P4 esophagus and LES that was stained with X-gal and neutral red. NT-3 expression in the circular muscle of the forestomach extended into the LES. (E) Confocal microscope image of an oblique section through the abdominal esophagus at E17.5 that was immunostained for β-galactosidase. β-galactosidase-LIR was restricted to the epithelium, thus confirming the X-gal staining of this tissue layer. The weaker staining in the outer muscle layer was tissue autofluorescence as it also occurred when the primary antibody was omitted and it was not stained by X-gal (A-C). Scale bar in C = 100 μm and also applies to A,B and D. Scale bar in E = 100 μm. Blood vessel, bv; epithelium, ep; lower esophageal sphincter, les; muscle wall, mw.

Fig. 2. NT-3 expression, detected as β-galactosidase-LIR, confirmed detection of β-galactosidase protein with X-gal staining that had been observed in the smooth muscle layers of the GI organs and some blood vessels (See Fig. 1 and subsequent figures for examples of X-gal staining patterns). In contrast, X-gal staining observed within the mucosa/submucosa at E17.5 and P4 was not confirmed by β-galactosidase-LIR in any of the organs studied, with the exceptions of the esophageal epithelium (Fig. 1E) and weak staining in the lamina propria of the stomach antrum and corpus (A). Confocal images of examples of the β-galactosidase-LIR in smooth muscle shown here include

the stomach and a GI blood vessel (A), small intestine (B), and large intestine (C). Scale bar in A = 100 μm and also applies to B and C. Circular muscle, cm; lamina propria, lp; longitudinal muscle, lm; mucosa, muc; myenteric plexus, mp; villi, vi.

Fig. 3. NT-3 expression patterns in the stomach wall at E12.5 (A,B), E13.5 (C,D), and E15.5 (E,F). Photomicrographs of X-gal-stained stomach sections (A,C,E) are shown on the left and matching sections counterstained with neutral red to their right (B,D,E). Initially, at E12.5, NT-3 was expressed in the mesenchyme, and by E13.5 as this tissue began to differentiate, strong expression became largely restricted to the smooth muscle layers and weaker expression to the lamina propria. In contrast, there was no expression in the epithelium, which is especially clearly shown by comparison of the insets in A and B (1.6X enlargements of portions of A and B). By E15.5 most tissue layers could be clearly and consistently identified and NT-3 expression was completely restricted to the smooth muscle layers and lamina propria. Scale bars = 100 μm; those in B, D and F, apply to A, C and E, respectively. Liver, li; mesentery, mes; submucosa, sub; other abbreviations as in Figs. 1 and 2.

Fig. 4. NT-3 expression patterns in the smooth muscle layers of the antrum, pylorus and duodenum. Photomontages of X-gal-stained sections of antrum, pylorus and anterior duodenum (A,C) are shown immediately above matching sections counterstained with neutral red (B,D). As illustrated here at E15.5 (A,B) and E17.5 (C,D), NT-3 expression in the antrum occurred in both the circular and longitudinal smooth muscle layers and rapidly transitioned to expression throughout the smooth muscle of the pylorus, which was dominated by a thick circular muscle, and then rapidly transitioned to expression that was restricted to the circular muscle layer of the

duodenum. Scale bars = 100 μm; the bar in B also applies to A. Abbreviations as for Figs. 2 and 3.

Fig. 5. NT-3 expression patterns in the stomach at P4. Photomontage of the corpus-forestomach transition region stained with X-gal and neutral red (A), and photomicrographs of X-gal-stained sections of antrum (B) and forestomach (D) above matching sections counterstained with neutral red (C,E). At P4, similar to earlier ages, NT-3 was expressed in the circular and longitudinal muscle layers of the antrum and some regions of the corpus and only in the circular layer of the remaining regions of the corpus. At the border between the corpus and forestomach there was a rapid transition from expression in both smooth muscle layers to expression only in the circular muscle of the forestomach (A). NT-3 expression patterns are shown at higher magnification for the antrum (B,C) and forestomach (D,E). Blood vessel walls in the submucosa that expressed NT-3 (B,C) are indicated by arrowheads (C). Scale bars = 100 μm; those in C and E apply to B and D, respectively. Abbreviations as for Figs. 2 and 3.

Fig. 6. NT-3 expression in the intestines within the abdomen (A,B) or the intestinal sac (C,D) at E12.5, and in the small intestine within the abdomen at E13.5 (E,F). Photomicrographs of X-gal-stained intestine sections (A,C,E) are shown on the left and semi-adjacent sections counterstained with neutral red to their right (B,D,E). The section in F was taken at the level of the bile duct. Within the abdomen at these ages, some regions of the intestines consisted of mesenchyme and epithelium (A,B intestine on left; C,D), whereas in other regions the muscle wall had begun to differentiate (A,B intestine on right). NT-3 expression was present in the mesenchyme when it was the predominant tissue layer. As the muscle wall formed it also became the site of the bulk

of NT-3 expression. Weak expression occurred in the remaining mesenchyme. Scale bars = 50 μm; those in B, D and F apply to A, C and E, respectively. Abbreviations as for Figs. 1-3.

Fig. 7. NT-3 expression in the small intestine at E15.5 (A,B) and E17.5 (C,D) was restricted to the circular smooth muscle layer. Photomicrographs of X-gal-stained intestine sections (A,C) are shown above (A), or on the left (C) of semi-adjacent sections counterstained with neutral red (B and D, respectively). By E15.5 villi had formed in the small intestine. The weak X-gal stain present in the villi at E17.5 (C,D) was most likely an artifact as this tissue was not stained at E15.5 (A,B) and at E17.5 it did not exhibit β-galactosidase-LIR (Fig. 2B). Scale bar in D = 100 μm and it applies to A-C. Abbreviations as for Figs. 2 and 3.

Fig. 8. NT-3 expression in the small intestine at P4 (A-C) was restricted to the circular smooth muscle layer. Photomicrographs of an X-gal-stained section of small intestine (A) is shown above an adjacent section counterstained with neutral red (B), which is above an enlarged image (C) of the lower left portion of the section in B. The weak X-gal stain present in the villi was most likely an artifact as discussed in the legend to Fig. 7. A blood vessel that expressed NT-3 (arrowhead) and a portion of the bile duct were present in these sections. Scale bars = 50 μm and the bar in B also applies to A. Abbreviations as for Figs. 2, 3 and 6.

Fig. 9. NT-3 expression in the cecum at E15.5 (A,B), E17.5 (C,D) and P4 (not shown) was similar to that observed in the small intestine – largely restricted to the circular smooth muscle layer. Photomicrographs of X-gal-stained cecum/intestine sections (A,C) are shown above semi-adjacent sections counterstained with neutral red (B,D). The

example illustrated at E15.5 includes the junction of the large intestine with the cecum, whereas at E17.5 the junction of the small intestine (ileum) with the cecum is shown. Mesenteric blood vessels that were cross-sectioned in (D) are indicated by arrowheads. All but one of these vessels were present in (C) where their NT-3 expression can be observed. Scale bar in D = 200 μm and it also applies to A-C. Large intestine, lg intest; other abbreviations as for Fig. 2.

Fig. 10. NT-3 expression patterns in the large intestine at E13.5. Photomicrographs of X-gal-stained large intestine sections are shown on the left (A,C,E) and adjacent (B and F) or semi-adjacent (D) sections counterstained with neutral red to their right. NT-3 expression in the large intestine within the abdomen of some embryos at E13.5 (A-F) occurred throughout the mesenchyme (A,B), but in other specimens at some anterior-posterior levels (C-F) the outer muscle wall had begun to differentiate, and in parallel NT-3 expression became largely restricted to this tissue layer. There was no expression in the epithelium. Scale bars = 50 μm; the bar in D also applies to A-C and the bar in F also applies to E. Abbreviations as for Figs. 1-3.

Fig. 11. NT-3 expression patterns in the large intestine at E15.5. Photomicrographs of X-gal-stained large intestine sections (A,C) are shown to the left of semi-adjacent sections counterstained with neutral red (B,D). NT-3 expression in the large intestine at E15.5 (A-D) occurred in the circular smooth muscle layer (A-D), and was equally strong in (C), weaker in (D), or absent from (A,B) different segments of the longitudinal smooth muscle layer. By E15.5 folding of the mucosa had begun. Scale bar in D = 100 μm and it applies to A-C. Mesentery, mst; other abbreviations as for Figs. 2 and 3.

Fig. 12. NT-3 expression patterns in the large intestine at E17.5. Photomicrographs of

X-gal-stained large intestine sections (A,C) are shown to the left of semi-adjacent sections counterstained with neutral red (B,D). Similar to the expression pattern at E15.5, NT-3 expression in the large intestine at E17.5 occurred in the circular smooth muscle layer (A-D) and was equally strong in (C,D), weaker in, or absent from different anterior-posterior levels of the longitudinal smooth muscle layer. By E17.5 formation of the mucosa was largely complete. Scale bar in B = 200 μm and in D = 50 μm and they also apply to A and C, respectively. Abbreviations as for Fig. 2.

Fig. 13. NT-3 expression pattern in the large intestine at P4. Photomicrographs of X-gal-stained large intestine sections (A,C) are shown to the left of semi-adjacent sections counterstained with neutral red (B,D). At P4, the predominant NT-3 expression in the large intestine occurred in the circular smooth muscle layer (A-D). At this age the mucosa appeared mature. Scale bar in B = 200 μm and in D = 50 μm and they also apply to A and C, respectively. Abbreviations as for Fig. 2.

Fig. 14. NT-3 expression in mesenteric blood vessel walls at E15.5 (A,B) and E17.5 (C,D) are illustrated. Photomicrographs of X-gal-stained sections containing GI organs and mesentery (A,C) are shown to the left of adjacent sections counterstained with neutral red (B,D). Blood vessels present in B and D are indicated by arrowheads and the NT-3 expression in their walls can be observed at similar locations in (A) and (C), respectively. The insets in (C) and (D) are 2.2X magnifications of the cross-sectioned blood vessel indicated by the arrowhead in (D). Scale bar in B = 100 μm and in D = 50 μm; they also apply to A and C, respectively. Mesenteric attachment, mst att; small intestine, sm intest; stomach, stom; other abbreviations as for Figs. 2, 10 and 13.

Fig. 15. Confocal microscope images, illustrating the apparent lack of NT-3 expression (β-galactosidase-LIR) in myenteric ganglia at E17.5. Each image in each of the four panels (A-D) is oriented with the circular muscle on the left, the myenteric plexus/ganglia in the middle and longitudinal muscle on the right. (A) NT-3 expression (left panel; stained with FITC-labeled secondary antibody) and myenteric neurons that exhibited HuC/D-LIR (middle panel; stained with RRX-labeled secondary antibody; arrows) did not co-localize (right panel; merge of NT-3 expression and HuC/D-LIR). Example shown here is from the stomach wall. (B) NT-3 expression (left panel; stained with RRX-labeled secondary antibody) and undifferentiated neural crest cells (neuronal and glial precursors) that exhibited p75-LIR (middle panel; stained with FITC-labeled secondary antibody; arrows) did not co-localize (right panel; merge of NT-3 expression and p75-LIR). Example shown here is from the small intestine wall. (C) NT-3 expression (left panel; stained with RRX-labeled secondary antibody) and glial cells that exhibited S100-LIR (middle panel; stained with FITC-labeled secondary antibody; arrows) did not co-localize (right panel; merge of NT-3 expression and S100-LIR). Example shown here is from the large intestine wall. (D) NT-3 expression (left panel; stained with RRX-labeled secondary antibody) and ICC-MYs that exhibited c-Kit-LIR (middle panel; stained with FITC-labeled secondary antibody; arrows) did not co-localize (right panel; merge of NT-3 expression and c-Kit-LIR). In addition to ICC-MYs, some cells in the muscle layers expressed c-Kit, but also did not co-localize with NT-3 expression. Example shown here is from the stomach wall. Scale bars in A-D = 10 μm.